Variation in resistance to parasitism in aphids is due to symbionts not host genotype

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Natural enemies are important ecological and evolutionary forces, and heritable variation in resistance to enemies is a prerequisite for adaptive responses of populations. Such variation in resistance has been previously documented for pea aphids (*Acyrthosiphon pisum*) attacked by the parasitoid wasp *Aphidius ervi*. Although the variation was presumed to reflect genotypic differences among the aphids, another potential source of resistance to *A. ervi* is infection by the facultative bacterial symbiont *Hamiltonella defensa*. Here, we explored whether variation among symbiont isolates underlies variation among *A. pisum* clones in resistance to *A. ervi*. Although maternally transmitted, *H. defensa* is sometimes horizontally transferred in nature and can be experimentally established in clonal aphid lineages. We established five *H. defensa* isolates in a common *A. pisum* genetic background. All of the five isolates tested, including one originating from another aphid species, conferred resistance. Furthermore, isolates varied in levels of resistance conferred, ranging from 19% to nearly 100% resistance. In contrast, a single *H. defensa* isolate established in five different aphid clones conferred similar levels of resistance; that is, host genotype did not influence resistance level. These results indicate that symbiont-mediated resistance to parasitism is a general phenomenon in *A. pisum* and that, at least for the isolates and genotypes considered, it is the symbiont isolate that determines the level of resistance, not aphid genotype or any interaction between isolate and genotype. Thus, acquisition of a heritable symbiont appears to be a major mode of adaptation to natural enemy pressure in these insects.

defense | endosymbiont | γ-proteobacteria | mutualism | *Wolbachia*

Vertically transmitted bacterial symbionts are widespread in invertebrates (e.g., refs. 1–5), but, in the vast majority of instances, the role of the symbiont in particular host–symbiont interactions remains unknown. Because vertically transmitted microbes largely depend on host reproduction for transmission, any benefit conferred to the host that increases host survival or fecundity relative to their uninfected counterparts enhances symbiont transmission within host populations (6). Most benefit-conferring symbioses that have been characterized in invertebrates are nutritional (e.g., refs. 1, 2, and 7). For instance, insects that feed only on nutrient-limited substrates (e.g., plant sap or blood) often harbor mutualistic microorganisms that supply nutrients lacking in their diets. Nutritional interactions, however, represent only one of several potential types of beneficial symbioses. Among other roles, symbionts can bestow the ability to avoid or overcome attack from natural enemies. Relative to microorganisms, animals are metabolically constrained, and they can benefit from microbial synthesis of substances that aid in their defense. Two recently discovered examples of symbiont-mediated defense are found in the staphylinid beetle *Paederus* and the marine bryozoan *Bugula*; in both, bacterial symbionts produce toxic polyketides that confer protection against predation (8–11).

Recent studies of the symbionts of *Acyrthosiphon pisum* (the pea aphid) also support the idea that symbionts may exert diverse effects on their host's phenotype (12–16). Pea aphid populations around the world are known to harbor at least five vertically transmitted (mother to offspring) facultative ("secondary") symbionts (SS) in addition to the obligate primary symbiont *Buchnera aphidicola*. Although the nutritional function of *Buchnera* is relatively well understood (17, 18), the roles of these SS in *A. pisum* are only now coming to light. *Regiella insecticola* (formerly the U-type or PAUS) has been implicated in host–plant specialization in Japanese *A. pisum* (ref. 16; but see ref. 19), and *Serratia symbiotica* (R-type or PASS in these studies) has been implicated in thermal tolerance in North American *A. pisum* (13, 14). Most relevant to the current study is the finding that isolates of *S. symbiotica* and *Hamiltonella defensa* (T-type or PABS) SS confer partial resistance to parasitoid wasps (15).

Resistance of insect hosts to parasitoid attack is widespread (20). Resistance is often mediated by insect host hemocytes that encapsulate parasitoid eggs (e.g., refs. 21–23), but encapsulation is not the only means of resistance. Aphids rarely encapsulate parasitoid eggs, yet numerous studies have documented that *A. pisum* clones vary greatly in resistance to parasitism by an important natural enemy, the solitary endoparasitic wasp, *Aphidius ervi* (e.g., refs. 24–30). Such variation in resistance to parasitism is commonly considered a function of the host genotype (reviewed in ref. 31). We previously found that single isolates of two *A. pisum* SS, *S. symbiotica* and *H. defensa*, conferred partial resistance to parasitoid attack in a common genetic background (15), indicating that at least some of *A. pisum*'s variation in resistance to parasitism is attributable to heritable symbionts rather than to the aphid nuclear genome. This view is corroborated by the work of Ferrari and colleagues (32), who found a correlation between the presence of *H. defensa* (called PABS in that study) among *A. pisum* clones and resistance to attack from *A. ervi* and its congener *Aphidius eadyi*.

These results do not indicate the extent to which variation in *A. pisum* resistance is caused by variation in the symbiont isolate, the host nuclear background, or interactions between isolate and host background. Little variation within *S. symbiotica* and *H. defensa* has been found in the 16S rDNA sequence (33) or in two other protein-encoding genes (34), yet bacteria that display little divergence at orthologous genes often impose very different phenotypes on their hosts. For example, isolates of the arthropod reproductive parasite *Wolbachia* that are identical at 16S rDNA have been shown to cause very different phenotypes (35).

In this study, we seek to determine whether the symbiont-mediated resistance phenotype is a general phenomenon in *A. pisum*/*A. ervi* interactions. We examine multiple *H. defensa* isolates in a common genetic background of *A. pisum* to determine whether the resistance phenotype is a general property of *H. defensa* in *A. pisum* and whether isolates differ with respect to levels of resistance conferred. We also examine the resistance phenotype of one particular *H. defensa* isolate in

Abbreviation: SS, secondary symbionts.

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multiple aphid genetic backgrounds to determine whether interactions between symbiont and host genotype influence the expression of resistance.

Materials and Methods

Study System. *A. pisum*, accidentally introduced to North America from Europe at around 1870, is a polyphagous pest of legumes, including forage crops, such as clover and alfalfa, and vegetables, such as peas and lentils (36, 37). This aphid is cyclically parthenogenetic in much of its range. Reproduction is asexual during the summer, and, in response to a decreasing photoperiod in autumn, sexual forms develop and produce eggs that overwinter on their host plants (38). In the laboratory parthenogenetic clones may be maintained indefinitely. Our *A. pisum* cultures are maintained as separate clones, each descended from a single parthenogenetic female. All clones are maintained on caged *Vicia faba* (fava bean) and held in an environmental chamber at 20°C ± 1°C and on a 16:8 light/dark cycle.

The five types of SS that are found at intermediate frequencies include three phylogenetically distinct γ-proteobacterial lineages: *S. symbiotica*, *H. defensa*, and *R. insecticola* (39–42), a *Rickettsia* (α-proteobacteria) (39), and a *Spiroplasma* (Mollicutes) (43). The γ-proteobacterial symbionts have only recently been formally named (34) and were previously referred to by multiple provisional labels. *S. symbiotica* has been called the R-type SS, S-sym, or PASS (39, 41, 44), *H. defensa* has been called the T-type SS or PABS (40, 45), and *R. insecticola* has been called U-type and PAUS (40, 46).

A. ervi (Haliday) (Hymenoptera: Braconidae), also introduced to North America from Europe, is a solitary endoparasitoid (47). The adult female wasp lays an egg inside its aphid host. The egg hatches, and the resulting larva feeds and develops inside the living aphid over a period of 5–8 days, eventually killing the host and causing the aphid cuticle to stretch and harden, a process that results in an aphid "mummy." This intimate physiological and biochemical association between endoparasitoid and aphid provides an opportunity for interactions between host defenses, SS, and developing wasp larvae. *A. ervi* was collected in Tompkins County, NY, in 2000 and is now in continuous culture in the M.S.H. laboratory (University of Arizona) on *A. pisum* clone 5A (from Wisconsin), which does not harbor SS.

Establishment of Experimental Lineages. We used a microinjection technique (15, 44) to experimentally manipulate SS infection status, thus allowing us to study the effects of a particular SS in comparison with others or with uninfected aphids, all with the same host genetic background. To ensure that aphid cultures were not contaminated, we verified the nuclear genotypes of experimental lineages with a diagnostic fingerprinting technique (intersequence simple repeats) (40, 48). Diagnostic PCR was used to verify the stability of SS composition (40). Diagnostic PCR primers used for *H. defensa* were (T1279F CGAGGGAAAGCGGAACTCAG and 35R CTTCATCGCCTCTGACTGC). Diagnostic PCR was conducted at 10-μl volumes using a standard reaction mix and PCR conditions as in Sandström *et al*. (40). The densities and location of *S. symbiotica* in artificially infected aphids are similar to those found in naturally infected aphids (15), and we generally expect artificially infected lineages to be very similar to natural counterparts with respect to SS density and localization. Parasitism assays (see below) were conducted a minimum of 15 generations after the artificial inoculation procedure to allow SS densities to approach equilibrium within the aphid host.

Generality of SS-Mediated Resistance in Multiple *A. pisum* Clonal Lineages. To determine whether the same *H. defensa* isolate generates similar resistance effects in multiple aphid backgrounds, we artificially inoculated five uninfected *A. pisum* clones with a *H. defensa* isolate obtained from aphid clone NY1 (New York) (isolate 8–2b in ref. 33). We previously found that this NY1 *H. defensa* conferred a 43% reduction in successful parasitism by *A. ervi* in a single aphid clone (5A) (15). In addition to this NY1→5A lineage, which has been maintained in the laboratory for 4 years without loss of SS (verified with diagnostic PCR), we infected four additional clonal lineages of *A. pisum* (7A, UT-A, UT-B, and UT-C) with *H. defensa* from aphid clone NY1. These artificially infected lineages are named NY1→7A, NY1→UT-A, NY1→UT-B, and NY1→UT-C, respectively (Table 1). For logistical reasons, resistance assays for the five treatments were conducted in two experiments. In the first experiment, NY1→7A and NY1→5A were assayed in comparison with their corresponding uninfected clonal lineage (i.e., 7A and 5A). In the second experiment, NY1→UT-A, NY1→UT-B, and NY1→ UT-C were assayed in comparison with their corresponding uninfected clonal counterparts (i.e., UT-A, UT-B, and UT-C) (Table 1).

Table 1. Creation of experimental lineages

H. defensa isolate	Uninfected recipient clone	New clonal lineage
NY1	5A (WI)	NY1 → 5A
NY1	7A (NY)	NY1 → 7A
NY1	UT-A	NY1 → UT-A
NY1	UT-B	NY1 → UT-B
NY1	UT-C	NY1 → UT-C
UT1	5A	UT1 → 5A
UT2	5A	UT2 → 5A
UT3	5A	UT3 → 5A
NY1	5A	NY1 → 5A
Ac1 (AZ)	5A	Ac1 → 5A

Shown are multiple uninfected *A. pisum* clones infected with single *H. defensa* isolate (NY1) (first five rows) and multiple *H. defensa* isolates infecting the same uninfected clone (5A) (last five rows). *H. defensa* isolates are from *A. pisum*, except Ac1, which was from *A. craccivora*. AZ, Arizona; WI, Wisconsin; NY, New York.

Resistance Effects of Different *H. defensa* Isolates in the Same *A. pisum* Clonal Lineage. We also investigated the role of different *H. defensa* isolates transferred into the same aphid genetic background (clone 5A). In addition to the 5A clonal lineage already artificially inoculated with the *H. defensa* isolate from clone NY1, we created three additional 5A clonal lineages with *H. defensa* isolates from three additional *A. pisum* clones (UT1, UT2, and UT3), resulting in experimental lineages UT1→5A, UT2→5A, and UT3→5A, respectively. We also inoculated clone 5A with *H. defensa* isolated from another aphid species, *Aphis craccivora*, to yield clone Ac1→5A (Table 1). Again, for logistical reasons, we conducted two experiments to perform the resistance assays for all five treatments. In the first experiment, we compared the resistance phenotype of the UT1→5A, UT2→5A, and UT3→5A lines to that of SS-free lineage 5A. In the second experiment, we compared NY1→5A and Ac1→5A to their uninfected counterparts.

Resistance Bioassays. The susceptibility of our artificially inoculated lineages to parasitism was measured with an assay modified from Henter and Via (24) and used in Oliver *et al.* (15). By using cages consisting of modified polystyrene cups inverted over potted *V. faba* plants, we confined 30 second-instar *A. pisum* nymphs 20–24 h before wasp introduction. Just before the experiment, wasps were given oviposition experience by exposing them to uninfected aphids. Females with oviposition experience were then individually assigned at random to the control

Table 2. Logistic regression analyses of resistance effect of the NY1 *H. defensa* isolate in different *A. pisum* genotypes

Assay	Regression equation	β_1	β_2
NY1 → 5A vs. 5A	$Y = 1.66 + 0.84^{NY1}$	$P < 0.0001$; 95% CI 0.63–1.05	n/a
NY1 → 7A vs. 7A	$Y = 1.12 + 0.60^{NY1}$	$P < 0.0001$; 95% CI 0.43–0.77	n/a
NY1 → UT-A vs. UT-A	$Y = 0.20 + 0.45^{NY1}$	$P < 0.0001$; 95% CI 0.24–0.66	n/a
NY1 → UT-C vs. UT-C	$Y = 0.21 + 0.40^{NY1}$	$P = 0.0007$; 95% CI 0.17–0.64	n/a
NY1 → UT-B vs. UT-B	$Y = 0.27 + 0.53^{NY1}$	$P < 0.0001$; 95% CI 0.34–0.72	n/a
5A vs. 7A	$Y = 1.86 + 0.14^{7A}$	$P = 0.21$; 95% CI −0.08 to 0.36	n/a
NY1 → 5A vs. NY1 → 7A	$Y = 0.42 - 0.09^{NY1\text{-}7a}$	$P = 0.25$; 95% CI −0.25 to 0.07	n/a
UT-C vs. UT-A vs. UT-B	$Y = 0.69 - 0.04^{UT\text{-}A} + 0.12^{UT\text{-}B}$	$P = 0.78$; 95% CI −0.28 to 0.20	$P = 0.33$; 95% CI −0.12 to 0.36
NY1 → UT-C vs. NY1 → UT-A vs. NY1 → UT-B	$Y = -0.23 - 0.018^{NY1\text{-}UT\text{-}A} - 0.03^{NY1\text{-}UT\text{-}B}$	$P = 0.89$; 95% CI −0.26 to 0.22	$P = 0.80$; 95% CI −0.27 to 0.21

Comparing artificially inoculated lineages to their genetically identical counterparts (first five assays) and comparing resistance effects among *H. defensa*-infected lineages and among uninfected lineages (last four assays). The regression equation is $Y = \beta_0 + \beta_1 X_1 + \ldots \beta_p X_p$. CI, confidence interval; n/a, not applicable.

or one of the experimental lineages. We removed wasps from arenas after 6 h. Arenas (caged colonies of exposed aphids) were incubated at 20°C ± 1°C and a 16:8 light/dark cycle. After 10 days, we counted the numbers of surviving aphids and mummies to determine susceptibility to parasitism. In susceptible aphids, mummies are almost always found within 8 days after parasitism. However, to ensure that particular treatments did not result in significantly delayed wasp development, we also examined several cup cages for each treatment for mummies at days 12 and 14 postparasitism. Using JMP-IN 4.0 statistical software, we analyzed the proportion of successfully parasitized aphids in a logistic regression framework.

Results

Does a Single *H. defensa* Isolate Confer Resistance to Parasitism in Multiple *A. pisum* Clonal Lineages?. In all five *A. pisum* host backgrounds, the NY1 *H. defensa* isolate significantly increased resistance to parasitism by *A. ervi* (Table 2 and Fig. 1). Logistic regression analyses indicate that reductions in successful parasitism for *H. defensa*-infected aphids ranged from 32–41% in the first experiment, and from 45–56% in the second experiment. Furthermore, in each of the two experiments, degrees of resistance did not vary significantly among clones infected with the NY1 isolate (Table 2). Mean resistance varied by 9% among infected lines in the first experiment and by only 3% in the second experiment. In addition, levels of resistance to parasitism by *A. ervi* did not vary significantly among uninfected clones in each of the two experiments (Table 2). The greatest mean difference in resistance between uninfected clones in the same experiment was 13%.

Do Different *A. pisum H. defensa* Isolates Confer Resistance to Parasitism by *A. ervi* in a Common Aphid Genetic Background? All four *H. defensa* isolates originating from *A. pisum* conferred resistance to parasitism by *A. ervi* in *A. pisum* clone 5A (Table 3 and Fig. 2), with logistic regression analyses indicating reductions in successful parasitism ranging from 29% to 82%. Interestingly, the levels of resistance conferred varied significantly among different *A. pisum*-derived *H. defensa* isolates in the same aphid background (Table 3 and Fig. 2*B*). In particular, the *H. defensa* isolate from *A. pisum* clone UT1 conferred extremely high levels of resistance. Only 6% of the aphids in this line (UT1→5A) succumbed to parasitism. In the second experiment, the *H. defensa* Ac1 isolate from a different aphid species, *A. craccivora*, also conferred resistance to parasitism by *A. ervi* in *A.*

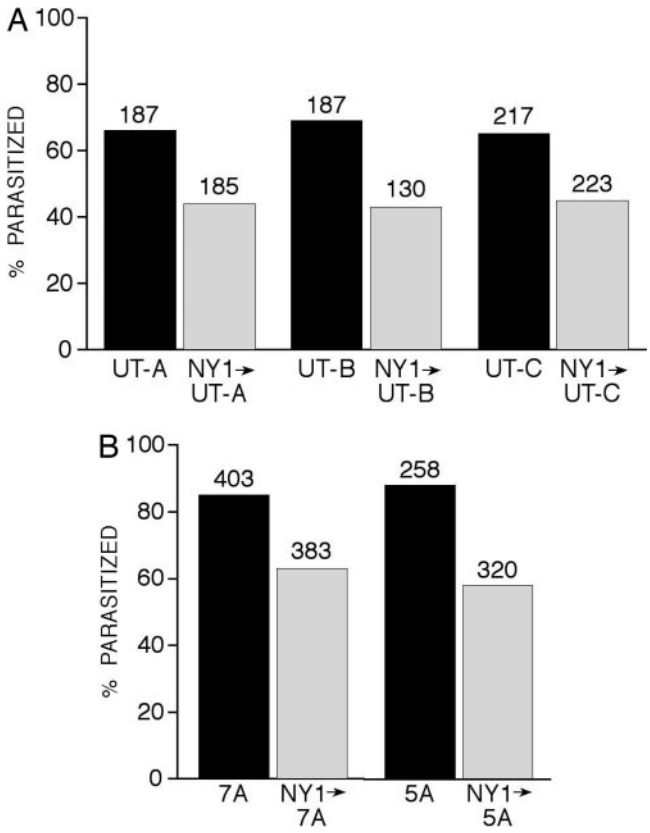

Fig. 1. Proportion of *A. pisum* successfully parasitized by *A. ervi*. Each treatment in these graphs represents different *A. pisum* clones, each harboring the same *H. defensa* isolate (NY1). *A* and *B* correspond to separate experiments with different *A. pisum* clones. Numbers above the bars represent the total number of aphids examined (alive plus parasitized).

Table 3. Logistic regression analyses of the resistance effect of multiple *H. defensa* isolates in a single *A. pisum* clonal lineage (5A)

Assay	Regression equation	β_1	β_2	β_3
UT1 → 5A vs. 5A UT2 → 5A vs. 5A UT3 → 5A vs. 5A	$Y = -2.04 + 1.66Hd^{UT3} + 1.70Hd^{UT1} + 0.35Hd^{UT2}$	$P < 0.0001$; 95% CI 1.44–1.88	$P < 0.0001$; 95% CI 1.50–1.93	$P = 0.0003$; 95% CI 0.16–0.54
NY1 → 5A vs. 5A Ac1 → 5A vs. 5A	$Y = 0.61 + 0.64^{Ac1} + 0.93^{NY1}$	$P < 0.0001$; 95% CI 0.47–0.81	$P < 0.0001$; 95% CI 0.76–1.1	n/a
UT3 → 5A vs. UT1 → 5A vs. UT2 → 5A	$Y = -0.83 - 0.90Hd^{UT1} + 1.80Hd^{UT2}$	$P < 0.0001$; 95% CI −1.14 to −0.66	$P < 0.0001$; 95% CI 1.57–2.04	n/a
NY1 → 5A vs. Ac1 → 5A	$Y = 0.61 - 0.28Hd^{NY1}$	$P = 0.001$; 95% CI −0.46 to −0.10	n/a	n/a

Comparison of resistance effect of the artificially inoculated lineages to genetically identical counterparts (first two assays) and comparison of resistance effect among *H. defensa* isolates (last two assays). The regression equation is $Y = \beta_0 + \beta_1 X_1 + \ldots \beta_p X_p$. CI, confidence interval; n/a, not applicable.

pisum lineage 5A (Fig. 2*B*). The Ac1 isolate and NY1 isolate also conferred significantly different levels of resistance (Table 3).

Discussion

Symbiont-mediated resistance to parasitism appears to be a general phenomenon in the herbivorous insect *A. pisum*. We found that a single isolate of *H. defensa* from *A. pisum* conferred partial resistance to parasitism by *A. ervi* in five distinct aphid genetic backgrounds (Table 2 and Fig. 1). In addition, four different *H. defensa* isolates, acquired from distinct *A. pisum* clones, all conferred resistance to parasitism by *A. ervi* in a single aphid clonal background (Table 3 and Fig. 2). The same result was found for a fifth *H. defensa* isolate transferred from *A. craccivora* (also attacked by aphidiine braconid parasitoids), suggesting that the defensive role of *H. defensa* extends to other host species. Thus, multiple *H. defensa* isolates confer resistance, irrespective of genetic background of the *A. pisum* clone in which they are found. This finding complements a correlative study by Ferrari *et al.* (32) in which clones with *H. defensa* were more likely to be resistant to parasitism by *A. ervi* and its congener *A. eadyi*. Although the *H. defensa* isolates all conferred resistance in our study, levels of resistance were highly variable, ranging from 19% to nearly 100% resistance (Table 3 and Fig. 2*A*). Such variation has been noted in another system in which the symbiont provides defense: the particular isolate of fungal endophyte in perennial ryegrass determines the level of resistance to weevil herbivory (49). In contrast, the levels of resistance conferred by the same *H. defensa* isolate (NY1) were similar in five different aphid genotypes (Table 2). With respect to resistance phenotype, we did not find strong interactions between symbiont isolate and host genetic background as reported in other systems (50, 51). These results indicate that the symbiont isolate is more important in determining the level of resistance than either aphid genotype or the interaction between isolate and aphid genotype, at least for the sample of aphid genotypes in this study.

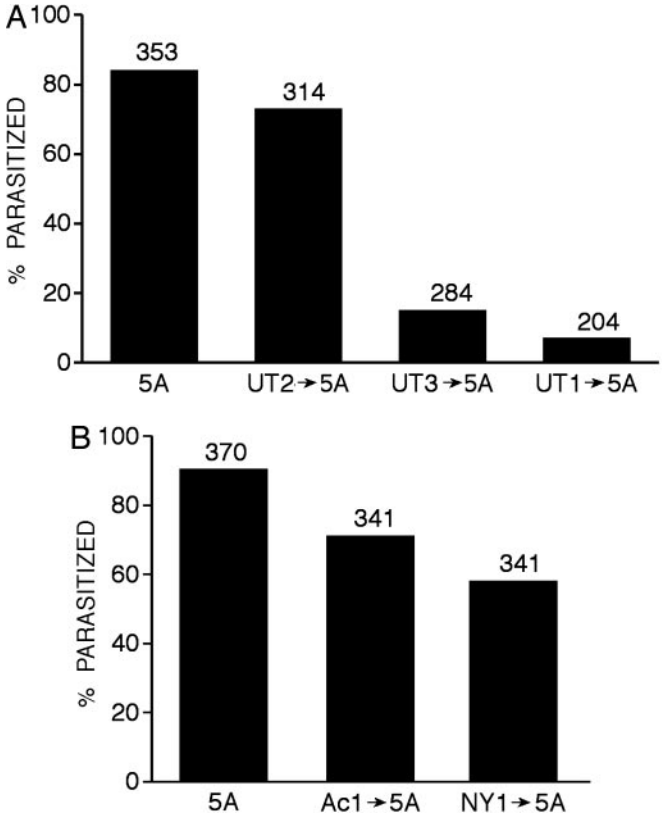

Fig. 2. Proportion of *A. pisum* parasitized by *A. ervi*. Each treatment in these graphs represents different lineages of the same *A. pisum* clone (5A), each with a distinct *H. defensa* isolate. *A* and *B* correspond to separate experiments with different *H. defensa* isolates. Numbers above the bars represent the total number of aphids examined (alive plus parasitized).

Although our experimental design does not allow us to compare the resistance levels of all uninfected clones used in this experiment, there do appear to be some differences in resistance to parasitism between the uninfected clones assayed in the first experiment [clones 5A (NY) and 7A (WI); raw mean, 86% susceptible] (Fig. 1*B*) and the uninfected Utah clones assayed in the second experiment (clones UT-A, UT-B, UT-C; raw mean, 66% susceptible) (Fig. 1*A*). However, even if these differences are real, they are small compared with the differences attributable to presence/absence or isolate of *H. defensa*. Differences in resistance also occur between species of SS that infect pea aphids; the previous study showed differences in level of resistance conferred by the NY1 isolate of *H. defensa* (called T-type in that study) and by an isolate of another symbiont species, *S. symbiotica* (R-type) (15).

Given these results, one might hypothesize that the bulk of the tremendous variation in resistance to parasitism by *A. ervi* found in *A. pisum* populations (24, 27) may be due to heterogeneity in aphid SS rather than aphid nuclear genes. The available sequence data for *H. defensa*, consisting of 16S rDNA sequences (33) and two other protein-encoding genes (34), show that isolates are closely related, with >99% sequence identity for orthologous genes.

In many pathogenic bacteria infecting humans, such as pathogenic *Escherichia coli* and *Salmonella enterica*, horizontally transferred genes, usually associated with bacteriophage, are the primary basis for variation in pathogenicity (e.g., ref. 52). All tested isolates of *H. defensa* possess bacteriophage (40, 53), and

these are a possible basis for genetic heterogeneity among isolates. This heterogeneity may, in turn, explain why isolates of *H. defensa* differ substantially in the degree to which they protect hosts from parasitism.

Little is known about the physiological mechanisms of parasitoid resistance in aphids, and nothing is known about the mechanism by which bacterial symbionts contribute to this resistance. Unlike many model systems in insect immunity, such as *Drosophila*, aphids rarely encapsulate parasitoids. In *A. pisum*, an encapsulation response to *A. ervi* appears very weak or nonexistent (K.M.O., personal observation). To describe *A. pisum–A. ervi* interactions, Falabella *et al.* (54) proposed a model in which the survival and growth of the parasitoid larva depends on the wasp successfully shifting the nutritional balance of the aphid host to favor the developing wasp larva. In susceptible aphids the parasitoid manipulates the bacteriocytes (aphid cells that harbor both primary and secondary symbionts) in ways which favor wasp growth (see also ref. 55). According to this model, resistant aphids may be those in which the manipulation is blocked and the parasitoid larva simply fails to thrive. Aphids may use defensive mutualisms with bacterial symbionts in lieu of or in combination with mechanisms based in the innate immune system, such as encapsulation. Host resistance via encapsulation in *Drosophila* is often costly (56–58), whereas no clear costs to infection with *H. defensa* have been demonstrated (59). As yet, nothing is known of genes underlying possible immune responses in aphids. Indeed, although >46,000 expressed sequence tags are now publicly available for *A. pisum*, very few show detectable homology to genes known to be involved in innate immunity, although such homologies can be found between the genes from humans, *Drosophila*, and nematodes. Possibly *A. pisum* exhibits a reduced or greatly modified immune system and is unusually dependent on symbiont-mediated defense.

Because no detrimental effects of *H. defensa* infection have been demonstrated to date (60), it is unclear why some *A. pisum* lineages are uninfected by *H. defensa* or other SS. The regular presence of uninfected lineages in natural populations implies that *H. defensa* is deleterious under some environments or that the rate of spontaneous loss of *H. defensa* from infected lineages is substantial. Under laboratory conditions, infections are extremely stable. After hundreds of generations of rearing, we have not observed loss of *H. defensa* from infected aphid clones, except in cases of double infections in which one of two symbiont types is eliminated. Thus, we hypothesize that *H. defensa* does impose a cost on its host under some conditions that occur regularly in natural populations but that have not yet been examined experimentally. Possibilities include starvation due to temporary removal from the host plant, poor-quality host plants, or extreme (high or low) temperatures, or passage through the sexual and egg stages of the life cycle.

A role of symbionts as agents in host defense is not limited to the *A. pisum–A. ervi* system. Examples of defensive mutualisms involving microorganisms can also be found in plants (60, 61) and marine and terrestrial arthropods (8–11, 62, 63). The *A. pisum–H. defensa–A. ervi* interaction is well suited to become a model system for studying symbiont-mediated resistance to natural enemies. The symbionts of *A. pisum* are among the best studied, and *A. pisum–A. ervi* interaction has already been studied from multiple perspectives, including behavior (e.g., refs. 64–66), population and community ecology (e.g., refs. 67–69), and the physiological aspects of the interaction (e.g., refs. 54 and 70–73). Understanding and appreciating symbiont-mediated resistance to parasitism also have important implications for biological control of herbivorous pests. The success of such programs clearly depends on the host population being susceptible to parasitoid attack. The variation in *A. pisum* resistance to parasitism due to SS may explain periodic failures of parasitoids to limit aphid abundance and damage in agricultural settings (37)

In this study, the primary source of the large observed variation in parasitoid resistance is symbiont infection. Another study showed that such variation in resistance does coincide with variation in reproductive output; parasitized aphids infected with *H. defensa* produce significantly more offspring than parasitized uninfected aphids (N.A.M. and M.S.H., unpublished data). Thus, acquisition of a particular secondary symbiont is a heritable change by which *A. pisum* lineages can lessen effects of attacking parasitoids. These results provide additional evidence that symbiosis can act as a source of rapid adaptive change during evolution.

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